Proc. Nat. Acad. Sci. USA
Vol. 71, No. 11, pp. 4536–4540, November 1974

Gap Junction Formation Between Reaggregated Novikoff Hepatoma Cells

(cell membranes/freeze-fracturing/electrical coupling)

ROSS JOHNSON, MARIE HAMMER, JUDSON SHERIDAN, AND JEAN-PAUL REVEL*

Department of Zoology, University of Minnesota, Minneapolis, Minn. 55455; and * Division of Biology, California Institute of Technology, Pasadena, Calif. 91109

Communicated by Don W. Fawcett, August 2, 1974

ABSTRACT **We have combined freeze-fracture and electrophysiological methods in a study of gap junction formation between reaggregated Novikoff hepatoma cells. Cell clumps are dissociated with EDTA, and the resulting single cells are allowed to reaggregate (5–180 min) in loose pellets in the presence of calcium at 37°. The earliest electron microscopic evidence for the genesis of new junctions is the appearance of flattened regions of the plasma membrane with a relative paucity of small intramembranous particles. These regions contain instead loosely organized groupings of 9- to 11-nm intramembranous particles, which are seen on the A face of the fractured plasma membrane, while corresponding pits occur on the membrane B face. We have termed the specialized membrane regions "formation plaques." They are seen as early as 5 min after reaggregation and are quite numerous by 30 min. Larger plaques are observed at later times. Plaques seen at 30 min are consistently matched with other plaques on apposed cells, although the extracellular space separating these structures still exceeds 10 nm. By 60 min, some matched plaques display a reduced extracellular space, resembling that of normal gap junctions. Between 30 and 60 min, aggregates of closely packed particles on A faces and hexagonally arranged pits on B faces frequently appear in the formation plaques. The aggregates, which are indistinguishable from small gap junctions, appear to enlarge over the subsequent 2-hr period as the number of unaggregated 9- to 11-nm particles declines. Microelectrode studies demonstrate progressive increases in the percent of interfaces containing low-resistance junctions and in the degree of electrical coupling in preparations incubated up to 2 hr. Coupling is first detected at about the same time as particle aggregates (or formation plaques with reduced extracellular spaces), and increases as aggregate sizes increase.**

It has been suggested that the direct diffusion of small molecules between the interiors of adjacent cells could be an important factor in cellular homeostasis and even the regulation of growth and differentiation (1–3). This exchange of molecules is believed to occur through gap junctions (4–7), which are specializations of two closely apposed membranes (8), each containing an aggregate of intramembranous particles (9, 10). The particles are presumed to bridge the narrowed extracellular space of a gap junction amd may provide an intercytoplasmic pathway for diffusing molecules (6, 10).

While many ultrastructural and physiological properties of gap junctions have been elucidated (1, 11, 12), we know relatively little about their formation. Electrophysiological studies indicate that formation of low-resistance connections (presumably gap junctions) can occur in a matter of minutes in certain developing systems (13–16) and in cell cultures (17–19). Previous ultrastructural studies suggest that gap junctions begin as small aggregates of intramembranous particles that gradually increase in size (20, 21). There is as yet little information about the details of the processes occurring within each junctional membrane or between membranes, i.e., across the extracellular space. Furthermore, there have been few attempts to correlate the appearance of low-resistance pathways with the development of gap junctions (17–19).

We report here a freeze-fracture and electrophysiological study of gap junction formation between Novikoff hepatoma cells. In suspension cultures these cells form clumps that contain typical gap junctions, display electrical coupling, and have pathways for the movement of small tracer molecules (5, 12). For our present investigation, we have studied junction formation as EDTA-dissociated cells were allowed to reaggregate. Preliminary reports of this study have been published (22–25).

MATERIALS AND METHODS

Dissociation and Reaggregation. Clumps of Novikoff hepatoma cells (N1S1-67) from suspension cultures (26) were dissociated as follows. Cells in logarithmic growth were centrifuged from standard growth medium (medium 67G, see ref. 26), resuspended in basal medium 42 (BM42, see ref. 26) + 10 mM EDTA at half their original concentration, and placed in a 37° New Brunswick G-27 gyratory shaker-incubator (200 rpm) for 15 min. After this EDTA treatment was repeated, cells were pelleted and resuspended in medium 67G at 1.5–3.0 times their initial concentration. A large proportion of the cells (91–96%) were present as single cells. Reaggregation was accomplished by centrifuging the disaggregated cells at 30 × *g* for 2.5 min and then incubating the shallow, loose pellets in medium 67G for 5, 15, 30, 60, 120, or 180 min at 37° in a 5% CO_2 atmosphere.

Electron Microscopy. Pellets of dissociated cells or reaggregated cells were fixed for freeze-fracturing with 2.5% glutaraldehyde in fresh culture medium (BM42) for 2 hr at room temperature. After treatment in 20% glycerol, also in medium BM42, the cells were frozen and fractured by reported procedures for the Balzers BA-360M freeze-etch device (27).

Electrophysiology. Loose pellets of reaggregated cells were broken apart in plastic petri dishes with fresh medium (67G) kept at room temperature (about 21°). Large clumps (20 to 50 cells), in which individual cells were distinguishable, were selected for electrophysiological study. Groups of cells within the clumps were checked for coupling, and the following types of data were obtained: (*i*) percent of coupled cell pairs, (*ii*) coupling coefficient (voltage in peripheral cells divided by voltage in cell injected with current), and (*iii*) input resistance (voltage in central cell divided by current injected). The apparatus and electrode preparation were similar to those described (16).

Every preparation was studied at room temperature and ambient CO_2 for 90 min after removal of the pellet from the incubator. Junctions continued to form during these study periods. However, the electrical data and electron microscopic observations indicate that the process was much slower under ambient conditions (about 1/10 the rate) than in the incubator (37°, 5% CO_2). In view of other possible sources of variability, the limited rate of junction formation under ambient conditions could be disregarded when the ultrastructural and electrophysiological data were compared.

RESULTS

Ultrastructure

Dissociated Cells. The membranes of dissociated, unreaggregated cells (Fig. 1) contain the usual patterns of intramembranous particles. A few gap junctions could be found, although far less frequently than in undissociated preparations. The observed junctions resemble undissociated junctions, rather than newly forming ones, and are thought either to link incompletely dissociated cells (see *Materials and Methods*) or to occur between cells and membrane fragments torn from cells during dissociation.

Development of "Formation Plaques." In contrast to the smooth contours of the membranes of freshly dissociated cells (Fig. 1), the membranes of cells in the early stages of reaggregation have an irregular appearance (Fig. 2). The first indication of the formation of new intercellular junctions is the appearance of smooth regions of the cell membrane associated with specific membrane particles (Fig. 2). Such "formation plaques" can already be seen in 5- and 15-min reaggregates. They are well defined by 30 min (Fig. 3). The formation plaques are characterized by (*a*) "flattened" areas of membrane averaging 0.3 μm in diameter, (*b*) clusters† of 9- to 11-nm intramembranous particles on membrane A faces (with pits on B faces, Fig. 4), and (*c*) the relative lack of intramembranous particles of smaller, more commonly observed sizes. At the earliest times the membranes of the formation plaques are not always as flat or as rich in 9 to 11-nm particles as they are at the later stages. There is a gradual increase in the average size and number of formation plaques in samples taken between 5 and 30 min. The plaques also increase in size between 30 and 60 min, and preliminary data, indicating that their number has decreased, suggest that they grow by fusion. One can in fact find examples of plaques at 60 min that appear to be in the process of fusion.

At 30 min many cell membranes have formation plaques that appear to be matched with similar plaques in the membranes of apposed cells. This can be seen in instances where the fracture plane leaves the membrane of one cell in the middle of a plaque, crosses the extracellular space, and propagates through the other membrane. In almost all cases at 30 min a plaque A face, with its characteristic 9- to 11-nm particles, is clearly matched with a pit-containing region on the apposed B face. The two membranes, however, are separated by an extracellular space typically in excess of 10 nm. The extent of matching at 5 and 15 min has not yet been determined because of the limited number of observed fracture breakthroughs. This leaves open the possibility that formation plaques appear independently in adjacent cells and become matched secondarily. In a single experiment, strikingly few formation plaques were found when cell contact was minimized by vigorous shaking during the 30-min incubation. This observation suggests that the development of formation plaques requires an interaction between adjacent membranes.

Appearance of Gap Junctions. At 30 min of reaggregation, the separation between membranes is typically in excess of 10 nm at the level of formation plaques (Fig. 5). However, in a few cases at 30 min, and more frequently at 60 min, the extracellular space between some plaques appears to be reduced (Fig. 6). In these instances, the proximity of the membranes resembles that found in normal gap junctions and the extracellular space typically widens at the edges of the matching plaques. At these times, aggregates† of closely packed intramembranous particles can be seen among the clustered 9- to 11-nm particles of the formation plaques. Correspondingly, a few regions of hexagonally arranged pits can be found in the midst of unordered pits on membrane B faces. The center-to-center distances for the particles in the aggregates (and for the highly ordered pits) range from 9 to 11 nm. At 60 min many of the plaques contain two or more aggregates (see Figs. 7 and 8), which are larger than those seen at 30 min. In plaques containing large aggregates, one often finds only limited numbers of free 9- to 11-nm particles. Where aggregates are found, the extracellular space is always observed to be of dimensions comparable to those of mature gap junctions. Since the aggregates, even at the earliest times, have all the features of small gap junctions, we use the two terms interchangeably in the following discussion.

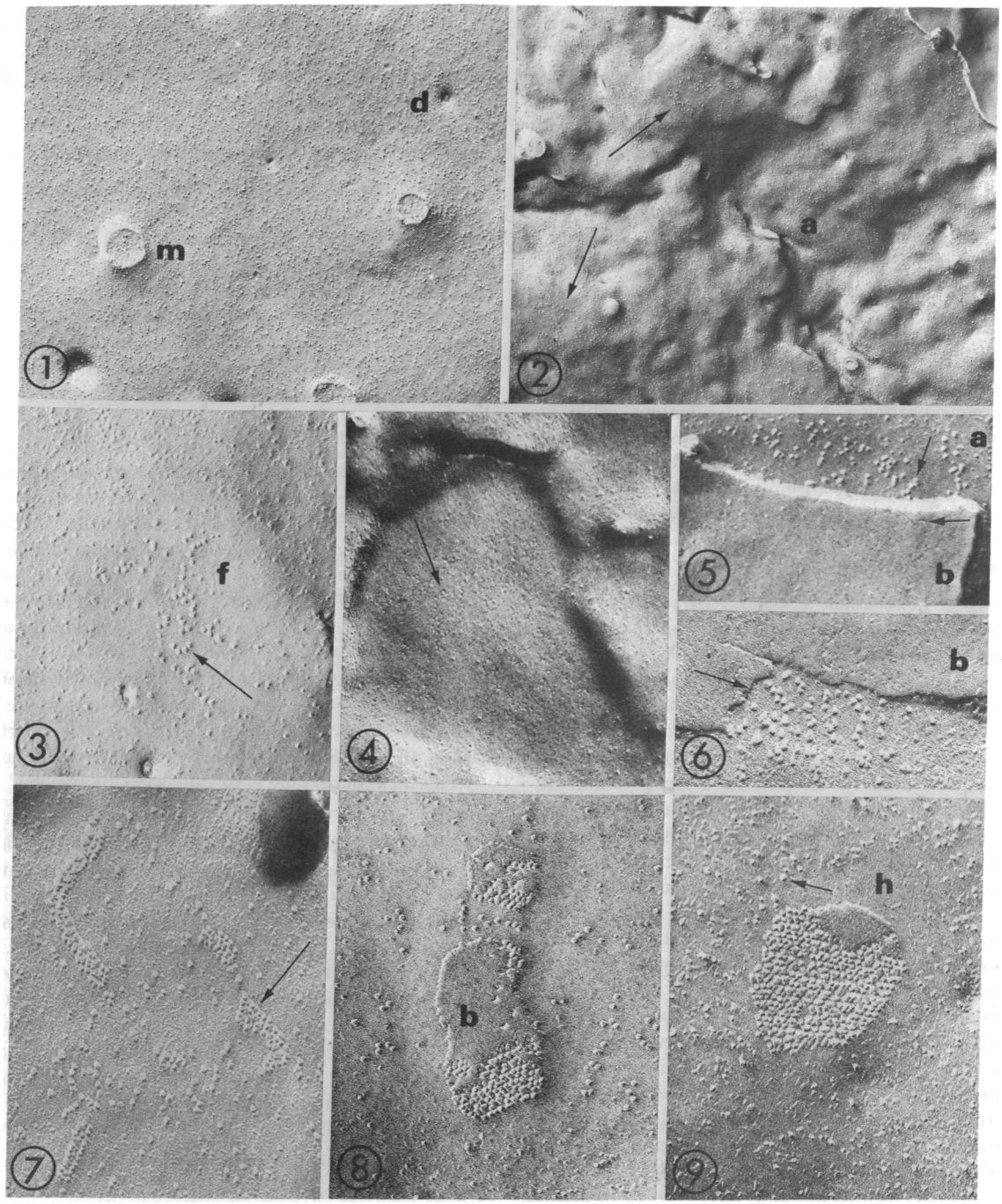
Later Stages in Gap Junction Formation. In samples examined at 120 and 180 min of reaggregation we find that the gap junctions have grown in size and have smoother outlines when compared to those seen at earlier times. By 180 min very few 9- to 11-nm particles can be observed either free around gap junctions (Fig. 9) or in separate formation plaques. The structure that was once seen as a wide formation plaque is seen by 120 and 180 min as a particle-free halo with reduced dimensions (compare Figs. 8 and 9). Gap junctions in undissociated Novikoff preparations are often larger than those seen at 180 min with even narrower particle-free halos, suggesting that junctional maturation and growth may continue beyond this time.

Electrophysiological observations

Parallel electrical measurements were made on clumps of cells broken from reaggregated pellets, and data are presented as a function of incubation time (Fig. 10). The probability that coupling can be found between a pair of adjacent cells (Fig. 10, top) increases with incubation time, the most rapid increase occurring over the 15- to 60-min incubation period. This results from an increase in the proportion of cell interfaces having low-resistance junctions, or in other words, from an increase in the number of direct low-resistance connections that a typical cell has with its neighbors. The quantitative relation between coupled cells and coupled interfaces (Fig. 10, top, right-hand scale) is derived from a probability argument (to be given in detail in a later publication). The proportion of interfaces with junctions is less than the proportion of coupled cells because coupling might occur directly between two cells and/or indirectly by way of other cells.

Accompanying the increase in the probability that two cells are coupled, there is an increase in the degree of coupling

† We have applied the term "cluster" to a number of particles of similar size grouped together without any obvious ordering. The term is used in contrast to "aggregate," which refers to a tight, often hexagonal, packing of particles.



FIGS. 1–9. Electron micrographs of freeze-fracture replicas of Novikoff hepatoma cells.

FIG. 1. Dissociated cell preparation. No unusual patterns of intramembranous particles are noted on this membrane A face. m, Microvilli; d, membrane depressions. ×41,000.

FIG. 2. 30-min reaggregate. This large A face (a) has a highly irregular topography and bears a number of formation plaques (arrows). ×41,000.

FIG. 3. 60-min reaggregate. This A face formation plaque is characterized by the flattened appearance of the membrane (f), clustered particles averaging 9–11 nm in diameter (arrow), and the relative lack of particles of more commonly observed sizes. ×103,000.

FIG. 4. 30-min reaggregate. Formation plaques are visualized on B faces as flattened areas with unordered pits (arrow). ×105,000.

FIG. 5. 30-min reaggregate. A matching of formation plaques in apposed cell membranes is illustrated by the clustered 9- to 11-nm particles (arrow) on the A face (a) and pits (arrow) on the B face (b). The white band between membrane faces represents an extracellular space exceeding 10 nm. ×100,000.

FIG. 6. 60-min reaggregate. Formation plaques in the apposed membranes are matched as in Fig. 5. However, the extracellular space is now reduced between the plaques (arrow) and widens on each side as in a mature gap junction. ×100,000.

FIG. 7. 60-min reaggregate. A tight packing of particles into several aggregates (arrow) is observed in the midst of free 9- to 11-nm particles. ×100,000.

FIG. 8. 60-min reaggregate. These aggregates are larger than those in Fig. 7, and a hexagonal packing is more apparent. Pitted B face (b) and reduced extracellular space are also shown. ×102,000.

FIG. 9. 120-min reaggregate. This gap junction, viewed on both A and B faces, has a broader particle-free halo (h) on the A face than junctions in undissociated cultures. Only a few 9- to 11-nm particles (arrow) are seen around the junction. ×100,000.

(given by the coupling coefficient, Fig. 10, middle). The increase over the 15- to 60-min incubation implies a decrease in average junctional resistance (i.e., increase in permeability to ions). The other alternative, i.e., an increase in nonjunctional resistance, is ruled out by the lack of an increase in the input resistance after 15 min of incubation (Fig. 10, bottom).

DISCUSSION

The studies that we report here on the formation of gap junctions suggest a possible sequence of events occurring within and between presumptive junctional membranes. They also provide information on the correlation between the formation of morphologically identifiable junctions and the development of low resistance intercellular pathways.

Ultrastructural Changes. The formation of gap junctions between reaggregating Novikoff cells appears to occur in stages, and we suggest that they occur in the following order: (*i*) development of formation plaques having a flattened membrane, a decrease in "small" intramembranous particles, and clusters of 9- to 11-nm particles; (*ii*) reduction of the extracellular space between matched formation plaques in adjacent cell membranes; (*iii*) aggregation of the 9- to 11-nm particles into tightly adherent groups, indistinguishable from small gap junctions; and (*iv*) growth of the small gap junctions, probably by addition of individual particles and fusion of small aggregates.

The 9- to 11-nm particles, which we believe ultimately make up the gap junction subunits, are the most conspicuous and consistent elements of the formation process and appear very soon after the cells begin to reaggregate. We feel that these particles may be involved in the initial attachment of the cells, in the production and coincidental matching of the formation plaques, and in the narrowing of the extracellular space between matched plaques. All of these effects would, in our view, ultimately depend on interactions of an extracellular component of the 9- to 11-nm particles, which may measure some 10 nm in length. We have no direct evidence for such extensions, but the 9- to 11-nm particles extend across the membrane bilayer, as evidenced by the B face pits, and in the fully developed junctions would be destined to form at least part of the extracellular subunits of the gap junction.

According to our working hypothesis, formation begins when the extracellular components of the 9- to 11-nm particles in the apposing membranes link up and contribute to the initial cell attachment. This interaction occurs initially across a space of 10–20 nm and determines the position at which the matched formation plaques will form. The interaction triggers certain changes in the adjacent membranes and initiates the development of the formation plaques. There is a flattening of the membranes and a displacement, or other change, in the smaller intramembranous particles, leading to their decreased densities in the developing plaques. These changes could be caused by phase changes in the lipids or alterations in submembrane elements, e.g., microtubules or microfilaments. After the plaques form, they accumulate additional 9- to 11-nm particles. These particles might come from membrane areas outside the plaque and accumulate because they have a greater affinity for the altered lipid phase and/or are guided by submembrane elements. Alternatively, the particles might be inserted directly into the membrane.

All of these processes, in our model, occur while the formation plaques are separated by an extracellular space of 10–20 nm. We suggest that this space is then reduced as a result of

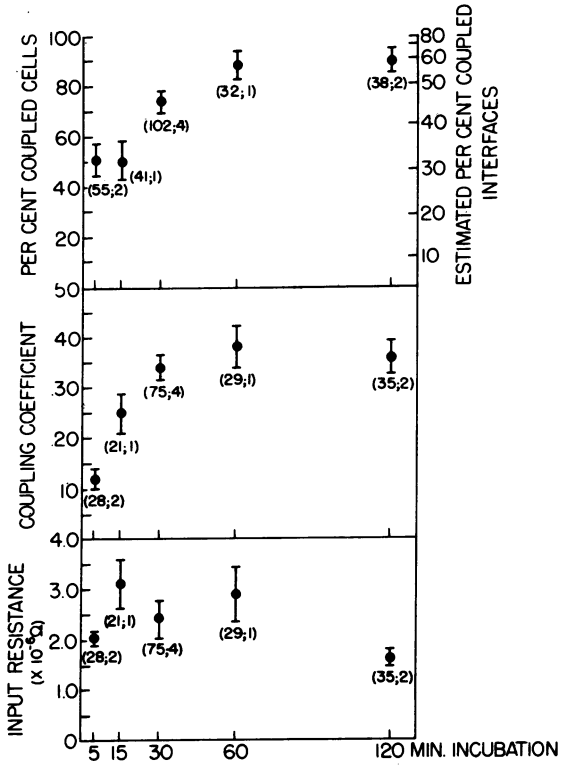


FIG. 10. Electrophysiological data from clumps of reaggregated cells. (Top) Percent of adjacent cells found to be coupled. Right-hand ordinate gives estimated percentage of coupled interfaces (see text). Mean ± SE; the latter was calculated from a formula for binomial distribution and probably underestimates the variance. (Middle) Coupling coefficients. Mean ± SE. (Bottom) Input resistances. Mean ± SE. All numbers in parentheses give total cell pairs studied and number of separate experiments. Common time scale for panels indicates the incubation times at 37°.

the cooperative interactions of progressively increasing numbers of 9- to 11-nm particles. This possibility is consistent with our finding (unpublished results) that the number of these particles per square micrometer in the formation plaques increases before the particles begin to aggregate.

After the reduction of the extracellular space, aggregates of particles appear in the plaques. We envision aggregating units as pairs of particles connected across the extracellular space. We suggest that these aggregates are formed from the 9- to 11-nm particles, although another class of particles could be specifically involved in formation. However, our suggestion is not only the most economical one, it is also consistent with the uniform occurrence of the aggregates in the midst of clustered particles, the gradual decrease in numbers of clustered 9- to 11-nm particles as the aggregates become more numerous, the similarity in size of the clustered and aggregated particles, and the observation of a 9- to 11-nm center-to-center spacing for particles in developing aggregates. The fact that center-to-center values for aggregated particles in untreated Novikoff cultures average slightly less than 9–11 nm may be due to technical reasons or to an actual alteration of the 9- to 11-nm particle in conjunction with or subsequent to aggregation. In other systems, particles have been observed near the edges of junctions which appear larger than the actual junctional particles (23, 28).

The growth of small aggregates into larger aggregates between 30 and 120 min probably occurs by further accumulation of single particles and by fusion with other aggregates. Features of many larger junctions between untreated Novikoff cells suggest that fusion may often be incomplete. Particle-free spaces can remain between associated aggregates, giving an appearance of "domains" (7) within an individual junction. Lack of complete fusion might also explain the fact that the roughly hexagonal lattices within adjacent domains are often out of register. Similar domains are found in gap junctions of other systems (7, 29, 30).

It is reasonable to question the relevance of our observations to junctional formation in other systems, since the Novikoff cells have been reaggregated artificially and are tumor cells. However, formation plaques with and without aggregates are seen in standard, nonreaggregating Novikoff cultures. Further, small aggregates have been observed in the early stages of gap junction formation in regenerating liver (21), as well as in embryos of amphibians (20) and chicks (23), where structures resembling formation plaques have also been reported.

Electrophysiological Correlation. Because the stages of formation and the appearance of electrical coupling do not occur synchronously in all cells, we have not yet been able to identify unequivocally the minimal structure(s) necessary for coupling. We feel, however, that formation plaques *per se* are probably unable to couple cells because the plaques are very numerous at times when the percentage of coupled cells is quite low. Although it would still be possible to argue that many formation plaques are necessary to couple a pair of cells (or that in some cases coupling might occur by way of matched formation plaques with a reduced extracellular space), it seems more likely that particle aggregates are the first sites of low resistance. There is a reasonable temporal correlation between the increases in percentage of coupled cells and degree of coupling, and the increases in number and sizes of particle aggregates. However, more quantitative data will be needed to confirm the role of the aggregates in coupling. Interestingly, it has been suggested recently that particle aggregation leads to the increased permeability of membranes in gap junctions (31).

In other studies to be reported in detail elsewhere we have demonstrated the transfer of fluorescein between 65% of the coupled cells in 30-min reaggregates. These findings suggest that at least some of the particle aggregates are permeable not only to small ions (i.e., have a low resistance), but also to larger molecules. Because we feel our method for detecting electrical coupling is more sensitive than our method for detecting movement of small amounts of dye, we feel it is possible that all aggregates with low electrical resistance are also permeable to fluorescein.

We thank Ms. Doris Preus for her excellent technical assistance, Drs. Miles Epstein and William Herman for commenting on the manuscript, and Ms. Lyn Steere for preparing the diagrams. Many of the electron microscopic studies were done by R.G.J. while he held a Visiting Associateship in Biology at Cal Tech. This work was supported by USPHS Grants CA11114, GM06965 and GM19224 and by the University of Minnesota Graduate School. J.D.S. holds a Career Development Award from National Cancer Institute, 5K04-CA70388.

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